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The structure of a pyoverdine produced by a *Pseudomonas tolaasii*-like isolate

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Abstract

Cultures of *Agaricus bisporus*, the most extensively cultivated mushroom, can be infected severely by *Pseudomonas tolaasii*. This pathogen is characterized by the so-called white line reaction, a precipitate formed on agar plates between its colonies and those of *P. reactans*, both belonging to the collective species *P. fluorescens*. A recent study has shown that a group of *P. tolaasii* isolates can be subdivided into two groups or 'siderovars', based on the pyoverdines they produce (Munsch *et al.* 2000). One group of strains is characterized by the pyoverdine described by Demange *et al.* (1990). A representative of the second group (strain Ps3a) was found to produce the same pyoverdine as a strain which had been classified before as *P. aureofaciens*. However, based mainly on 16S rRNA gene sequence comparisons and REP-PCR generated fingerprints, the two strains are not identical. They are also distinguishable from the *P. tolaasii* type strain.

Abbreviations: Common amino acids – 3-letter code; AcOHOrrn – N^5-acetyl-N^5-hydroxy-Orn; cOHOrn – *cyclo*-N^5-hydroxy-Orn (3-amino-1-hydroxy-piperidone-2); aThr – *allo*-Thr; Chr – pyoverdine chromophore; Suca – succinamide residue; MS – mass spectrometry; ESI – electrospray ionization; CA – collision activation; COSY – correlation spectroscopy; DEPT – distortionless enhancement by polarization transfer.

Introduction

The best known cultivated edible mushroom, accounting for more than one third of the world production, is *Agaricus bisporus* (in German speaking countries named 'Champignon'). A grave problem for the growers can be a bacterial infection that causes brown surface lesions referred to as blotch disease, making the crop unmarketable (Soler-Rivas *et al.* 1999). The bacterium responsible was described first by Tolaas (1915) as *Pseudomonas fluorescens.* After a serious breakout of the disease in England in 1918 it was investigated in detail by Paine (1918) and renamed in honor of the first author as *P. tolaasii*. *P. tolaasii* can be characterized by a highly specific peculiarity: when on an agar plate both a colony of *P. tolaasii* and one of certain other *Pseudomonas* spp. referred to as '*P. reactans*' are grown in a distance of about 1 cm from each other, a white line develops between these two colonies (Wong & Preece 1979; Preece & Wong 1982). Strains of '*P. reactans*' were identified as belonging either to biotype G according to the classification of Stanier *et al.* (1966) (= biovar V, Palleroni 1984) or being intermediate between *P. fluorescens* and *P. putida* (Zarkower 1984). The white line is formed by interaction of tolaasin, the lipodepsipeptidic toxin of *P. tolaasii* NCPPB 1116 (Nutkins

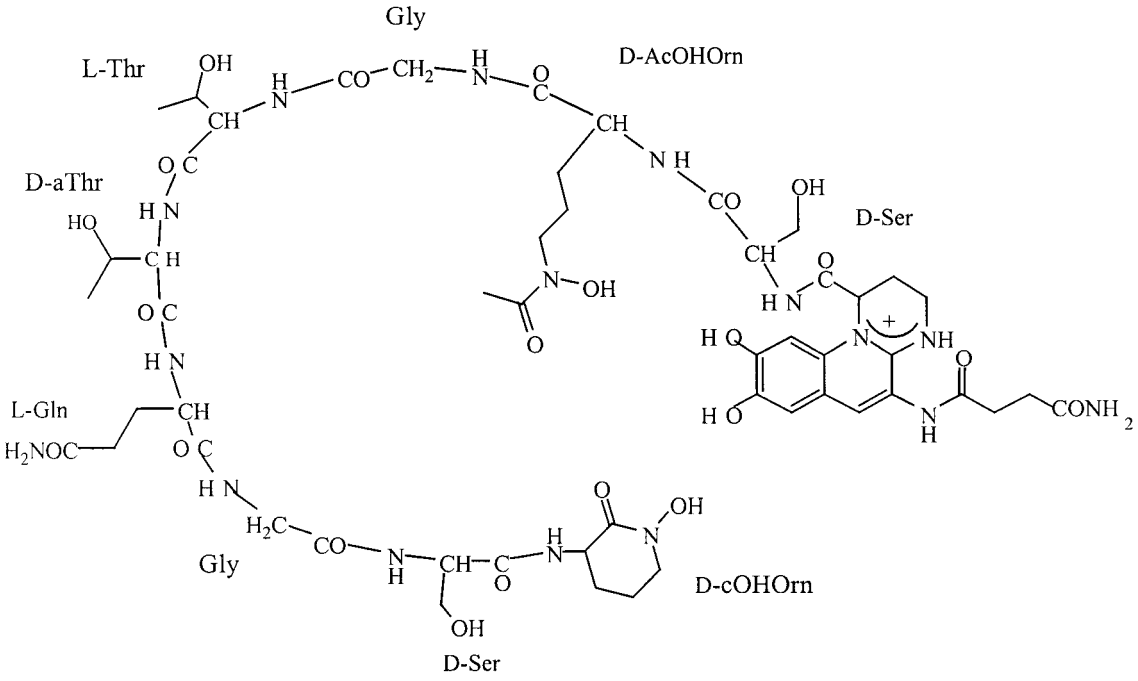

Fig. 1. Structure of the pyoverdin **II**.

et al. 1991) with a lipodepsipeptide isolated from '*P. reactans*' NCPPB 387 (Mortishire-Smith *et al.* 1991).

P. tolaasii was placed originally as *P. fluorescens* biotype B (= biovar II), but apparently the blotching isolates fit better into biotype G (= biovar V) (Zarkower *et al.* 1984). Biovar V (biotype G) of *P. fluorescens* is a rather ill-defined 'miscellaneous' group especially as far as the nutritional patterns are concerned. It seemed, therefore, to be of interest to see whether isolated siderophores could be used to establish relationships on a safer basis (cf. Meyer 2000). *P. fluorescens* belongs to the 'fluorescents' of the rRNA homolgy group I of the Pseudomonadaceae producing peptidic siderophores named pyoverdines (Elliott 1958; Budzikiewicz 1997). They are composed of a dihydoxyquinoline chromophore (for its structure see **II**) linked to a peptide chain comprising 6 to 12 L- as well as D-amino acids, some being partially modified. The peptide sequence is responsible for the recognition of the siderophore at the cell surface, but its composition may also be used for classification purposes (e.g., Budzikiewicz *et al.* 1997; Georgias *et al.* 1999; Meyer 2000).

Experimental

For bacterial growth, isolation of the pyoverdine, spectroscopic methods, total hydrolysis and amino acid analysis see e.g. Voss *et al.* (1999). Siderotyping methods including isoelectrophoresis and pyoverdine-mediated $^{59}Fe^{3+}$-uptake were performed as described previously (Meyer *et al.* 1998).

Results and discussion

In a comprehensive study it was shown that bacteria corresponding to the description of *P. tolaasii* could be grouped into two siderovars, *i.e.* strains producing different pyoverdines (Munsch *et al.* 2000). Siderovar 1 is characterized by the pyoverdine **I** which had been obtained from *P. tolaasii* NCPPB 2192^T (a white line producer, Zarkower *et al.* 1984). Its structure was established by Demange *et al.* (1990) as

Suca-Chr-D-Ser-L-Lys-L-Ser-D-Ser-L-Thr-D-Ser-L-AcOHOrn-L-Thr-D-Ser-L-cOHOrn (**I**).

We wish now to report the structure of the pyoverdine from the strain Ps3a, chosen as a representant of siderovar 2. Its molecular mass as determined by ESI-MS is 1276u. Amino acid analysis established

Table 1. Fragment ions of **II** obtained after CA by cleavage of the peptide bonds

Amino acid	N-terminal fragments		C-terminal fragments	
	n	B_n	*n*	Y''
Ser	1	442	9	–
AcOHOrn	2	616	8	–
Gly	3	673	7	662
Thr	4	774	6	605
aThr	5	875	5	504
Gln	6	1003	4	403
Gly	7	1060	3	275
Ser	8	1147	2	218
cOHOrn	9	–	1	131

B: X-NH-CHR-CO^+, Y": X'-CO-CHR-NH_3^+ (Roepstorff & Fohlman 1984).

the presence of L-Glu, Gly, L-Orn, D-Ser, L-Thr and d-aThr plus succinic acid. ^{1}H- and ^{13}C-NMR data including two-dimensional techniques (H,H-COSY, C,H-COSY, ^{13}C-DEPT) were identical with those reported for the pyoverdine **II** of a strain originally classified as *P. aureofaciens* (Beiderbeck *et al.* 1999). The sequence of the amino acids in the peptide chain except for the relative position of Thr and aThr (which can not be distinguished by MS or NMR) were determined by CA-MS (Table 1) as corresponding to that of **II**.

Suca-Chr-D-Ser-D-AcOHOrn-Gly-L-Thr-
D-aThr-L-Gln-Gly-D-Ser-D-cOHOrn (**II**).

Accordingly, pyoverdine-mediated $^{59}Fe^{3+}$-incorporation studies showed that strain Ps3a and the *P. aureofaciens* isolate reacted with an identical efficiency in cross-assays involving their respective pyoverdines, which were characterized by identical isoelectrophoresis patterns with three pyoverdine isoform bands at pHi 8.0, 5.3 and 5.0, respectively.

I and **II** have little in common except the large number of small neutral amino acids. The fact that a *P. aureofaciens* bacterium should produce the same pyoverdine (**II**) as a *P. tolaasii*-like isolate casted some doubt on the correct identification of the former, especially as the strain did not produce phenazine derivatives (see, e.g., Römer *et al.* 1979). Other experimental data supported the possibility that the *P. aureofaciens* strain had been wrongly identified originally. Thus, a 1492 nucleotide sequence representing almost all the 16S rDNA gene of the *P. aureofaciens* strain was determined (GenBank accession number AF 321239). The comparisons between AF 321239 and three available *P. aureofaciens* 16S rDNA sequences (AF 094722, PSEIAM07 and PAZ76656, respectively) revealed a high divergence between the present '*P. aureofaciens*' strain and the authentic ones.

In addition, the determination of phenotypic features with the API 100 system (Biomérieux) allowed to distinguish clearly the '*P. aureofaciens*' strain from strain Ps3a and from *P. tolaasii* LMG 2342^T. Also, REP-PCR studies performed according to De Bruijn (1992) revealed that the strain Ps3a and the '*P. aureofaciens*' strain do not belong to the same REP-PCR group, as highly diverse fingerprints were generated, distinguishing the two strains one from each other. Moreover, the '*P. aureofaciens*' strain failed to produce a white line towards a *P.* '*reactans*' reference strain and was seen as non-pathogenic on mushroom sporophores. Therefore, it should be considered as not physiologically related to brown blotch causal agents such as *P. tolaasii* or *P. tolaasii*-like bacteria.

The precise taxonomical assignment of '*P. aureofaciens*' has to be determined yet. The present study shows that strains producing identical pyoverdines could belong to different bacterial species. This was observed before for strains subsumed under the classical *P. fluorescens* conglomerate such as *P. fluorescens* ATCC 13525 and *P. chlororaphis* ATCC 9446 (Hohlneicher *et al.* 1995).

Conclusion

The structure analysis of the pyoverdines produced by bacteria corresponding to the description of *Pseudomonas tolaasii* according to pathogenicity and white line tests, confirmed the conclusion reached by siderotyping studies, as each siderovar corresponds to a specific pyoverdine structure. As the strain Ps3a is taxonomically distinguishable from the type strain of *P. tolaasii* and also from an isolate originally described as *P. aureofaciens*, studies are presently going on to precise the phylogenetical status of the strains. In any case, this is a further example illustrating the value of pyoverdine structures determination, together with siderotyping, for classification purposes.

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